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Genetics of the Serine Cycle in *Methylobacterium extorquens* AM1: Identification, Sequence, and Mutation of Three New Genes Involved in C_1 Assimilation, *orf4*, *mtkA*, and *mtkB*

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In a recent paper we reported the sequence of the beginning of a serine cycle gene cluster on the *Methylobacterium extorquens* AM1 chromosome, containing the genes encoding serine glyoxylate aminotransferase (*sgaA*), hydroxypyruvate reductase (*hprA*), and 5,10-methylenetetrahydrofolate dehydrogenase (*mtdA*) (L. V. Chistoserdova and M. E. Lidstrom J. Bacteriol. 176:1957–1968, 1994). Here we present the sequence of the adjacent downstream region containing three full and one partial open reading frames. The first of the full open reading frames (*orf4*) remains unidentified, while the other two (*mtkA* and *mtkB*) code for the two subunits of malate thiokinase, and the fourth, a partial open reading frame (*ppcA*), apparently encodes phosphoenolpyruvate carboxylase. Mutants containing insertion mutations in *orf4*, *mtdA*, and *mtdB* all were unable to grow on C_1 compounds, showing that these three newly identified genes are indispensable for the operation of the serine cycle. Mutants in *orf4* were also unable to grow on C_2 compounds, but growth was restored by glyoxylate, suggesting that *orf4* might be required for the conversion of acetyl coenzyme A to glyoxylate.

Methylobacterium extorquens AM1 is a pink-pigmented serine cycle methylotroph able to grow on methanol and methylamine as well as on a variety of multicarbon substrates (26, 31). While much progress has been made in the genetic studies of methanol and methylamine oxidation by *M. extorquens* AM1 and other methylotrophs (15, 20, 21), genetic studies of the serine cycle have started to develop more recently (2, 5–7, 13, 30). Up to now, four chromosomal regions of *M. extorquens* AM1 that encode serine cycle enzymes have been identified. One region contains a large cluster of serine cycle genes (Fig. 1), including genes necessary for the synthesis of serine glyoxylate aminotransferase (SGAT), *sgaA*; hydroxypyruvate reductase (HPR), *hprA*; 5,10-methylenetetrahydrofolate dehydrogenase (MTHFDH), *mtdA* (6); phosphoenolpyruvate carboxylase (PEPC), *ppcA* (2); and malyl-coenzyme A (CoA) lyase (MCL), *mclA* (13). Two other regions not linked to the large cluster mentioned above have been identified: one encoding enzyme(s) for the unknown acetyl-CoA oxidation pathway portion of the serine cycle and another encoding glycerate kinase (30). Little is known about these chromosomal regions. The fourth region, not linked to either of the above-described regions, contains the gene for serine hydroxymethyltransferase, *glyA* (7).

The goal of the present study was to perform genetic and physical analyses of the region of the *M. extorquens* AM1 chromosome located between *mtdA* and *ppcA*.

The *Escherichia coli* strains, DH5α (Bethesda Research Laboratories, Inc.) and S17-1 (29), used in this study were grown in LB medium in the presence of appropriate antibiotics as described by Maniatis et al. (23). *M. extorquens* AM1 was grown in the minimal medium described previously (13). Succinate (20 mM), methanol (100 mM), methylamine (20 mM), ethanol (40 mM) or ethylamine (20 mM) was used as the substrate. Methanol induction of mutants was carried out as described by Dunstan et al. (11). The following antibiotics at the concentrations indicated were used for *M. extorquens* AM1: tetracycline, 10 μg/ml; kanamycin, 100 μg/ml; and rifamycin, 50 μg/ml. The growth responses of mutants were tested on plates containing the substrates listed above in the presence or absence of a supplement of glyoxylate (1, 2, or 10 mM) or glycolate (20 mM). All the substrates, antibiotics, and supplements were purchased from Sigma. DNA-DNA hybridizations were carried out with dried agarose gels as described by Meinkoth and Wahl (24) at 68°C. For hybridizations, 6× SSC (1× SSC is 0.15 M NaCl and 0.015 M sodium citrate) was used, and 0.5× SSC was used for washes. Plasmid isolation, *E. coli* transformation, restriction enzyme digestion, ligation, blunting ends with T4 DNA polymerase, and filling in ends with Klenow enzyme were carried out as described by Maniatis et al. (23). All enzymes used in the molecular analysis of DNA were purchased from Boehringer (Mannheim, Germany). The chromosomal DNA of *M. extorquens* AM1 was isolated by the procedure of Saito and Miura (28). DNA sequencing was carried out with an Applied Biosystems automated sequencer at the University of California, Los Angeles, Sequencing Facility, for both strands. Translation and analyses of DNA and DNA-derived polypeptide sequences were carried out by using PC/Gene (Genofit SA, Geneva, Switzerland), DNAMaster (California Institute of Technology, Pasadena, Calif.), or the Genetic Computer Group programs. Enzyme activities were determined in *M. extorquens* AM1 crude extracts prepared as described previously (5). All measurements were done at room temperature in a total volume of 1 ml. The serine cycle enzyme activities were assayed as described previously (5). In addition, activity of malyl-CoA lyase was assessed by measurement of acetyl-CoA disappearance in the reverse reaction as described previously (14). All the substrates were purchased from Sigma. Spectrophotometric methods (19, 34) were used for protein determination. Triparental or biparental matings between *E. coli* and *M. extorquens* AM1 were performed overnight on nutrient agar. Cells were then washed with sterile medium and plated on selective medium at appropriate dilutions. In triparental matings, pRK2013 (10) was used as a helper plasmid. Rifamycin was used for *E. coli* counterselection.

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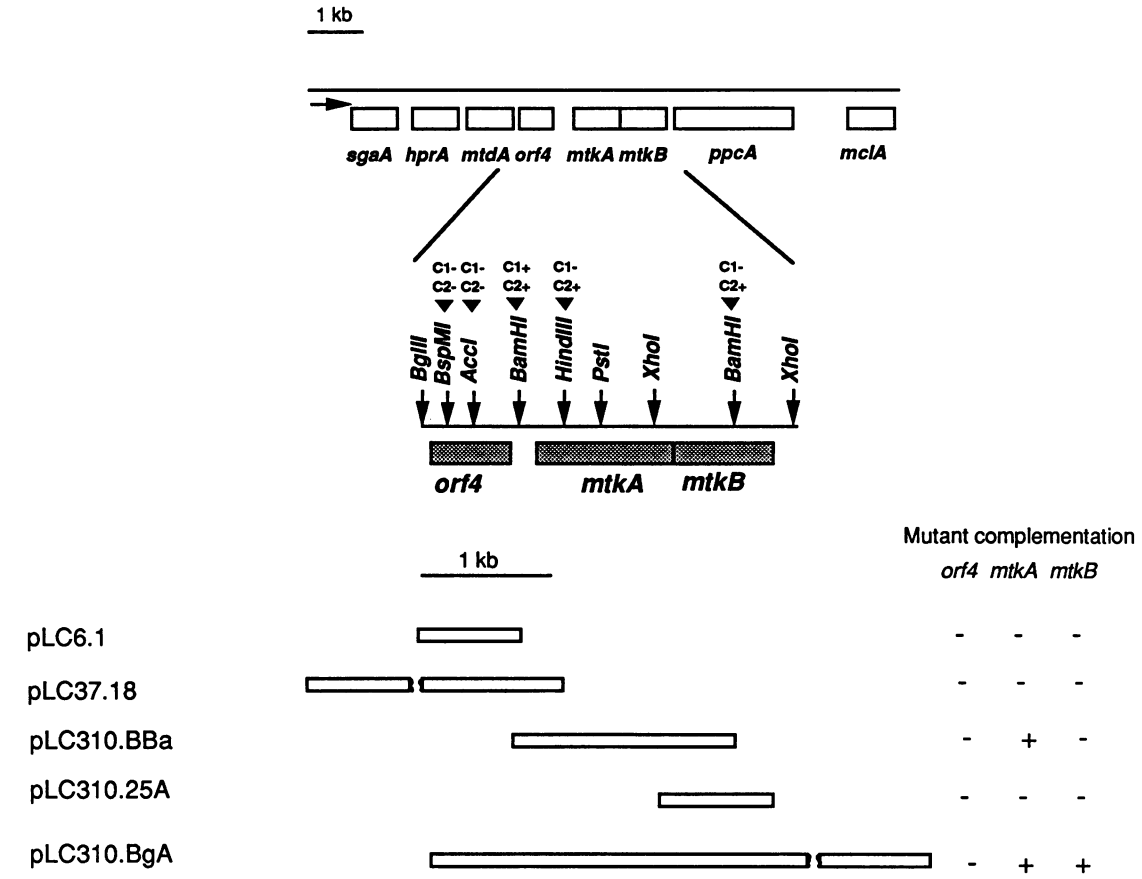


FIG. 1. Physical map of *M. extorquens* AM1 chromosomal region containing *orf4*, *mtkA*, and *mtkB* and results of mutant complementation with plasmids containing the indicated fragments. The horizontal arrow denotes the direction of transcription. Inverted triangles indicate sites at which insertion mutations were obtained in the course of this study. C1−, unable to grow on C_1 compounds; C1+, able to grow on C_1 compounds; C2−, unable to grow on C_2 compounds; C2+, able to grow on C_2 compounds.

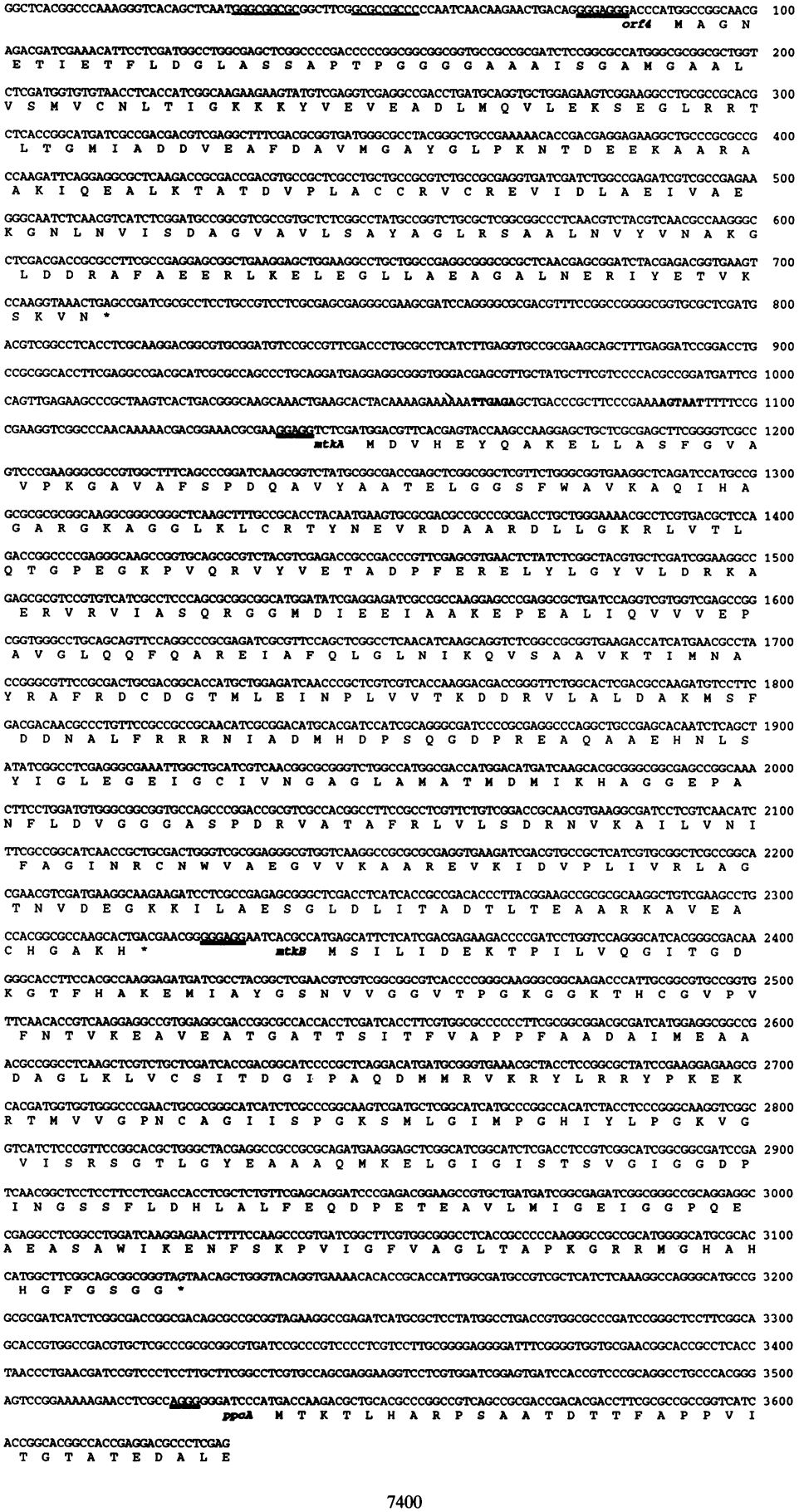
Sequencing of DNA fragment adjacent to *mtdA*. The *Eco*RI fragment of the *M. extorquens* AM1 chromosome (pBE7.21) containing the major portion of the gene for PEPC synthesis in its 3′ part was described previously (2). The 5′ part of this fragment was shown to contain truncated *hprA* (6). *mtdA* was identified downstream of *hprA* (6). We have sequenced the region between *mtdA* and *ppcA* in order to identify the corresponding genes and to determine their function in C_1 metabolism. A 3.6-kb *Bgl*II-*Xho*I fragment that spans this region (Fig. 1) was sequenced on both strands. The sequence of 26 nucleotides downstream of the *Bgl*II site has been published and shown to contain the 3′ end of *mtdA* (6). Figure 2 shows the sequence of 3,630 bp starting from the 27th nucleotide from the *Bgl*II site. The sequence analysis revealed the presence of three open reading frames and the 5′ terminus of a partial open reading frame, all transcribed in the same orientation coinciding with the orientation of transcription from the upstream genes *sgaA*, *hprA*, and *mtdA* and the downstream genes *ppcA* and *mclA* (2, 5, 6). The first open reading frame of 627 bp, including the stop codon, encodes a polypeptide with a calculated molecular mass of 21,726 Da. This open reading frame is separated from *mtdA* by a sequence of 88 bases. An inverted repeat sequence is found in the sequenced region, separating *mtdA* and the first open reading frame (calculated ΔG[25.5°C] of −27 kcal [ca.−113 kJ]), having the potential to form a stable stem-and-loop-type structure (underlined in Fig. 2).

The second open reading frame of 1,173 bases, including the stop codon, is located further downstream, separated from the first open reading frame by 432 bases. This second open reading frame is able to encode a polypeptide with a calculated molecular mass of 42,074 Da. A few copies of the GAAA motif found upstream of other methylotrophic genes (6) are present in this intergenic region. Sequences similar to the consensus −35 and −10 regions of σ^{70} promoters are present upstream of the second open reading frame (shown in bold in Fig. 2). It is not yet known whether they function as a promoter in *M. extorquens* AM1.

The third open reading frame (783 bases, including the stop codon), which could encode a polypeptide with a calculated molecular mass of 26,978 Da, is separated from the second open reading frame by 22 nucleotides. A fourth, partial open reading frame of 96 nucleotides is separated from the third open reading frame by 410 bases.

No known promoter sequences (9, 16, 22) or sequences similar to those of the putative *mxaF* promoter (21) were found between the third and fourth open reading frames or between *mtdA* and the first open reading frame.

Sequence comparisons to amino acid sequence deduced from first open reading frame. The amino acid sequence


orf4 M A G N
mtkA M D V H E Y Q A K E L L A S F G V A
mtkB M S I L I D E K T P I L V Q G I T G D
ppcA M T K T L H A R P S A A T D T T F A P P V I

derived from the first open reading frame was compared with sequences in GenBank. No significant similarity was found with known protein sequences. Analysis of the primary structure of the polypeptide translated from *orf4* for transmembrane helices by using the methods of Klein et al. (19a) and Rao and Argos (26a) (PC/GENE software) has shown that the polypeptide might be a membrane-associated protein, with a highly hydrophobic polypeptide stretch located at the beginning of the protein (amino acid residues 27 to 40).

Construction of insertion mutations in first open reading frame. In order to clarify the significance of *orf4* for C_1 metabolism, insertion mutations were made in the gene. The strategy for introduction of specific mutations into *orf4* was based on the exchange of DNA via homologous recombination in vivo between the wild-type gene and a gene that had been inactivated by an insertion mutation in vitro (27).

The 1-kb *Bgl*II-*Bam*HI fragment from pBE7.21 containing the entire *orf4* was cloned into pUC19 (Pharmacia) to generate pLC24.4, and a 1.4-kb DNA fragment from pUC4K (33) containing a Km^r gene was inserted into the *Acc*I site located approximately in the middle of the gene, so that the Km^r gene was transcribed in the same direction as *orf4*. The plasmid carrying the interrupted *orf4* (pLC24Sp1) was ligated with the suicide vector pAYC61 (4), and the resulting plasmid (pLC4Ac) was transformed into *E. coli* S17-1. The resulting *E. coli* strain was used as a donor in biparental matings with *M. extorquens* AM1, and the progeny were selected on succinate plates containing kanamycin. Of these progeny, 35% of the Km^r colonies were also Tc^s, which should be the result of a double recombination event leading to a complete gene replacement. DNA-DNA hybridization analysis using the *Bgl*II-*Bam*HI fragment containing *orf4*, the Km^r gene fragment, and the vector has shown that the mutants contained the insertion in the appropriate chromosomal location (data not shown).

The second specific mutation was introduced into the *Bsp*MI site at the beginning of *orf4*, starting again with pLC24.4. Plasmid pLC24BKm, containing the Km^r gene insertion into the *Bsp*MI site, was ligated with pAYC61; the resulting plasmid, pLC61.24B, was transformed into *E. coli* S17-1; and the resulting *E. coli* strain was used as a donor in matings with *M. extorquens* AM1. In this case, 30% of the Km^r progeny were also Tc^s. DNA-DNA hybridization analysis of a few representatives of these mutants confirmed the presence of the in vitro-mutated *orf4* at the proper location in the *M. extorquens* AM1 chromosome (data not shown).

Analysis of *orf4* insertion mutants. Growth responses were determined for 12 separate insertion mutant isolates carrying a mutation in *Acc*I and 10 mutants with insertions into *Bsp*MI. All of the mutants lost the ability to grow on C_1 compounds (methanol and methylamine) and C_2 compounds (ethanol and ethylamine), indicating that the *orf4* product is required for both C_1 and C_2 metabolism. Glyoxylate and glycolate have been previously used as supplements to characterize mutants that are unable to grow on both C_1 and C_2 compounds, which are defective in the unknown pathway of oxidation of acetyl-CoA to glyoxylate (12). The *orf4* mutants were unable to grow on either methanol or ethylamine in the presence of glycolate. Glyoxylate was able to restore growth of *orf4* mutants on ethylamine but not methanol.

Two of the *Acc*I insertion mutants, ORF4-1 and ORF4-2, and two of the *Bsp*MI insertion mutants, ORF4B4 and ORF4B7, were used for enzymological analysis. The levels of some key enzymes of the serine cycle (HPR, SGAT, PEPC, MTK-MCL, MTHFDH, and serine hydroxymethytransferase) were measured in these mutants grown on succinate and induced with methanol. All enzymes were present at levels similar to the wild-type levels (data not shown).

Expression of *orf4* in *M. extorquens* AM1. Two fragments containing *orf4* have been employed in efforts to complement *orf4* mutants. Both fragments were cloned into the conjugative plasmid pRK310 (10) in both orientations with respect to the *lac* promoter. The resulting plasmids were transferred into *orf4* mutants, ORF4-1, ORF4-2, ORF4B4, and ORF4B7. The smaller fragment used (1-kb *Bgl*II-*Bam*HI fragment, plasmids pLC6.1 and pLC310.24B [Fig. 1]) carried the entire *orf4*, the 3′ terminus of the upstream gene *mtdA*, and the region of 88 nucleotides separating the two genes but was unable to complement the mutants in either orientation with respect to the *lac* promoter. Therefore, a larger fragment was employed to exclude the possibility of expression problems. The larger fragment (5-kb *Sph*I-*Hin*dIII fragment, plasmids pLC37.18 and pLC37.19 [Fig. 1]) contained *hprA*, *mtdA*, and *orf4*, as well as the region upstream of *hprA* shown to contain a promoter for *hprA*, which is believed to be used also for transcription of *mtdA* (6). Transconjugants containing pLC37.18 or pLC37.19 were not able to grow either on C_1 or C_2 compounds, although the plasmids carrying cloned *orf4* were stably inherited in *M. extorquens* AM1 strains (data not shown).

Sequence comparisons of second and third open reading frames. The deduced amino acid sequence for the second open reading frame was compared with sequences in GenBank, and a high degree of similarity was found with the large subunit of succinyl-CoA synthetase (SCS) from *E. coli* (3) and *Thermus flavus* AT-62 (25). SCS catalyzes a reaction similar to the malate thiokinase (MTK) reaction in the serine cycle; so, the second open reading frame was a candidate for the gene encoding MTK, and we tentatively designated it *mtkA*. SCS is known to be a two-subunit enzyme (about 42 and 30 kDa). In *E. coli* and *T. flavus*, the two genes encoding the two polypeptides are organized in operons and translationally coupled (3, 25). MTK was purified from a facultative methylamine utilizer, *Aminobacter aminovorans* (32), and shown to be a two-subunit enzyme very similar to SCS biochemically (18). By analogy with the operon organization of the genes for bacterial SCS, it seemed possible that the third open reading frame might encode the second subunit of MTK, and it was tentatively designated *mtkB*. The amino acid sequence of the third open reading frame was compared with the sequences of the small subunit of SCS from *E. coli* and *T. flavus* AT-62 and mitochondrial SCS from rat liver (17), and a high degree of similarity was found between the four polypeptides. Large subunit polypeptides for the putative MTK and SCS are highly similar, sharing 50 and 43% identity with the β subunit of SCS from *E. coli* and *T. flavus*, respectively, with most nonidentical residues being conservative substitutions (data not shown). The most noticeable feature of the putative MTK small subunit polypeptide is that it is 33 residues shorter than the SCS small subunit polypeptides. Otherwise, the similarity is even greater for the

FIG. 2. Nucleotide sequence of 3,630-bp *M. extorquens* AM1 chromosome region containing *orf4*, *mtkA*, *mtkB*, and 5′ terminus of *ppcA*. Amino acid sequences are deduced from coding regions. Inverted repeat sequences are underlined, putative Shine-Dalgarno sequences are double underlined, the sequences bearing resemblance to the −35 and −10 regions of the σ^{70} consensus promoter are shown in bold, and asterisks indicate stop codons.

TABLE 1. Activities of some serine cycle enzymes in *mtk* mutants and transconjugants carrying *mtkA* and *mtkB*

Strain (plasmid)	Enzyme activity (nmol/min/mg of protein) with growth medium substrate indicated[a]:						
	MTK/MCL		MCL		PEPC[b] (MeOH)	HPR	
	Succ	MeOH	Succ	MeOH		Succ	MeOH
AM1 (wild type)	15	40	80	220	20	330	1,200
Hi19/Hi20[c]	0	0	37	150	17	300	1,000
MTKB1/MTKB2[c]	0	0[d]	58	130[d]	24[d]	300	1,200[d]
Hi20 (pLC310.BBa)	30	50	60	180	20	330	1,300
Hi20 (pLC310.BBb)	30	45	60	200	20	310	1,000
Hi20 (pLC310.BgA)	45	55	90	140	25	280	1,200
Hi20 (pLC310.BgB)	35	45	85	150	20	300	1,000
MTKB1 (pLC310.BgA)	35	50	65	180	20	350	950
MTKB1 (pLC310.BgB)	30	40	60	120	20	300	1,000

[a] Cells were grown on succinate (Succ) or methanol (MeOH), except as otherwise indicated. Data are the averages of two to four independent measurements. Values agreed within ±15%.
[b] PEPC activity was not measured in cells grown on succinate.
[c] Data are averages of activities measured in two mutants.
[d] Cells were grown on succinate, washed, and incubated with methanol for at least 20 h to allow for induction.

small subunits of putative MTK and SCS, sharing 60, 55, and 56% identity with *E. coli*, *T. flavus* AT-62, and rat liver β-subunit polypeptides, respectively (data not shown).

The sequence of 32 amino acids deduced from the partial open reading frame located downstream of *mtkB*, compared with sequences in the protein data bank, did not show considerable similarity to any known proteins. However, transposon mutagenesis data indicate that the region containing this open reading frame encodes PEPC (2). In addition, sequence data for the region located further downstream shows that it codes for a polypeptide showing strong similarity with the C termini of a number of PEPCs (8), and the sizes of these enzymes suggest that the partial open reading frame shown in Fig. 2 must code for the N terminus of PEPC; so, it has been designated *ppcA*.

Construction of insertion mutants in *mtkA* and *mtkB*. The 1-kb *Bam*HI-*Xho*I fragment from pBE7.21 containing the 5′ part of *mtkA* was cloned into pUC19 (pLC300). The *Hin*dIII site was removed from the pUC19 linker by cutting with *Hin*dIII, filling in ends by using the Klenow enzyme, and ligation, and the *Hin*dIII site of the insert of pLC300 was used for insertion of a 1.4-kb *Hin*dIII fragment from pUC4K carrying the Km^r gene. In the resulting plasmid (pLC303), the Km^r gene was transcribed in the same direction as *mtkA*. This plasmid was ligated into the *Kpn*I site of pAYC61, and the resulting plasmid (pLC17.6) was transformed into *E. coli* S17-1, which served as a donor strain in biparental matings with *M. extorquens* AM1. Km^r transconjugants were routinely selected on succinate-minimal medium plates. About 28% of the Km^r transconjugants were also Tc^s, the criterion for double recombinants. DNA-DNA hybridization analysis was used to confirm the presence of the mutation in the proper location on the chromosome of *M. extorquens* AM1. The *Bam*HI-*Xho*I fragment, used as a probe, hybridized to the 3-kb *Sph*I fragment in the wild-type chromosome and to the 4.4-kb fragment in the mutant chromosome (data not shown), confirming the presence of the mutation in the expected location.

For insertion mutations into *mtkB*, the 1.6-kb *Xho*I fragment containing *mtkB* was cloned into pUC19 (pLC1.2.1). The *Bam*HI site from the pUC19 linker was then removed by cutting with *Xba*I and *Kpn*I, blunting the ends with T4 DNA polymerase, and ligation, and the *Bam*HI site at the 5′ end of *mtkB* was used for insertion of the 1.4-kb *Bam*HI fragment from pUC4K containing the Km^r gene, resulting in plasmid pLC121XKm. The plasmid pLC121XKm was ligated with pAYC61, producing pLC61.121, and *E. coli* S17-1 carrying pLC61.121 was used as a donor in biparental matings with *M. extorquens* AM1. Km^r transconjugants of *M. extorquens* AM1 were selected on succinate-minimal medium plates and checked for their resistance to tetracycline. Approximately 60% of transconjugants showed a Tc^s phenotype, indicating a double recombination event. Two representatives of these were used for DNA-DNA hybridization analysis and shown to contain the Km^r gene cartridge in the proper location of the chromosome (data not shown).

An additional insertion mutation was made in the *Bam*HI site located in the region between *orf4* and *mtkA* (Fig. 1). The Tc^s mutants, which were shown by hybridization to be double crossover recombinants (data not shown), were able to grow on both C_1 and C_2 compounds. These mutants were not analyzed further, but they show that these insertion mutants should not have polar effects on downstream genes.

Analysis of *mtkA* and *mtkB* insertion mutants. Growth responses of mutants in *mtkA* and *mtkB* were checked on plates containing methanol, methylamine, ethanol, or ethylamine. All the mutants were unable to grow on C_1 compounds but grew normally on C_2 compounds, indicating that MTK is specifically involved in C_1 metabolism. Four mutant strains, Hi19, Hi20 (*mtkA*), MTKB1, and MTKB2 (*mtkB*), were used for enzymological analysis.

A method for detection of MTK activity based on measurement of ADP appearance was previously used for partially purified MTK from *A. aminovorans* (19). However, in cell extracts of *M. extorquens* AM1, high background activity was present with this method, and we were unable to detect MTK activity, as has been reported previously (1). Therefore, the combined activity of MTK-MCL was measured to assess the levels of MTK, and the MCL activity was measured in the reverse reaction, by acetyl-CoA disappearance (14). Combined MTK-MCL activity was absent in extracts of the mutants, while activities of MCL, PEPC, and HPR were present at the wild-type level, and a normal pattern of methanol induction was observed (Table 1).

Expression of *mtkA* and *mtkB* in *M. extorquens* AM1. Two DNA fragments were used to test for complementation of the *mtk* mutants, cloned into pRK310 in both orientations with respect to the *lac* promoter (Fig. 1). pLC310.BBa carried the 2.4-kb *Bam*HI fragment containing *mtdA*, its upstream region, and a truncated *mtkB* under the control of the *lac* promoter. The plasmid pLC310.BBb carried the same fragment in the

opposite orientation. Plasmids pLC310.BgA and pLC310.BgB carried the 5-kb *Bgl*II fragment containing both *mtkA* and *mtkB*, including the adjacent regions under the *lac* promoter and in the opposite orientation, respectively. These plasmids were transferred into *M. extorquens* AM1 in three-way matings, and the growth responses of the transconjugants were tested. pLC310.BBa and pLC310.BBb were both able to complement the *mtkA* mutants, and pLC310.BgA and pLC310.BgB were both able to complement the *mtkA* and *mtkB* mutants, indicating that a promoter must be present upstream of *mtkA*.

Coupled activity of MTK-MCL was measured in transconjugants carrying plasmids described above, and the level of activity was found to be at or slightly above the wild-type level (Table 1).

Concluding remarks. In this work we have continued the characterization of a cluster of serine cycle genes in *M. extorquens* AM1 that was initiated in our previous work (2, 5, 6, 13). The sequence of 3,630 nucleotides presented here completes the sequence of an almost 8-kb fragment of the *M. extorquens* AM1 chromosome carrying six coding regions (*sgaA*, *hprA*, *mtdA*, *orf4*, and *mtkAB*), all involved in C_1 assimilation. The functions of *sgaA*, *hprA*, and *mtdA* are clear, since they have been shown to encode, respectively, SGAT, HPR, and MTHFDH (6). Studies using the T7 expression system had shown that the region between *hprA* and *ppcA* encoded polypeptides with sizes of approximately 23, 43, and 34 kDa (2). The present studies show that they correspond to the gene products of *orf4*, *mtkA*, and *mtkB*, respectively.

The gene after *mtdA*, *orf4*, encodes a product that is apparently involved in the unknown pathway of biosynthesis of glyoxylate from acetyl-CoA, but the biochemical role of this gene product is unknown. So far in *M. extorquens* AM1, all of the mutants isolated that are unable to grow on both C_1 and C_2 compounds have been complemented by DNA fragments different from the one that contains this cluster of serine cycle genes (7, 30). Therefore, *orf4* is a newly identified gene required for growth on C_1 and C_2 compounds, and the *orf4* mutants must be defective in a previously unidentified function that overlaps in these types of metabolism. In addition, the phenotype of the *orf4* mutants is unusual in that glyoxylate restores growth only on C_2 compounds, not C_1 compounds, while in previously isolated mutants, glyoxylate restores growth on both C_1 and C_2 compounds (30). This phenotype suggests that the product of *orf4* must play a dual role in C_1 metabolism, and one of these roles is also involved in C_2 metabolism. A similar phenotype has been recently observed for *M. extorquens* AM1 mutants in *glyA*, the gene for serine hydroxymethyltransferase. In this case it seems that in addition to its well-known role in the serine cycle, this enzyme has a second role, required for both C_1 and C_2 metabolism (7). It is not known why it was not possible to complement the *orf4* mutants in *trans*. Since the *orf4* product is predicted to be a membrane protein, it is possible that truncated products are made in the mutants that are more competitive than wild-type proteins for membrane sites or somehow alter the function of the wild-type protein.

The genes *mtkA* and *mtkB* were found to encode the large (β) and the small (α) subunits of MTK. Both genes revealed a high degree of similarity with their counterparts encoding the α and β subunits of SCS in *E. coli* and *T. flavus*. In *E. coli*, *sucC* and *sucD*, encoding the β and α subunits of SCS, respectively, are parts of an operon composed of genes encoding enzymes of the citric acid cycle (3), while in *T. flavus* the corresponding genes, *scsB* and *scsA*, comprise an operon together with the gene encoding malate dehydrogenase (*mdh*) (26). MTK has been purified from the facultative methylotroph *A. aminovorans* and shown to be distinct from the SCS present in this organism, although both enzymes were able to catalyze the SCS reaction (18). The high degree of similarity found for the primary structure of MTK with primary structures of known SCSs confirms the close relatedness of these two enzymes, shown biochemically previously (18), and probably indicates their common origin.

The identification of these genes in *M. extorquens* AM1 is significant, since it has never been possible to demonstrate MTK in this strain (1). It is possible to infer MTK activity by assaying the joint activity of MTK and MCL and then assaying MCL separately (5), but it has never been proven that the enzyme MTK exists in *M. extorquens* AM1. We have shown convincingly that *mtkA* and *mtkB* encode the two subunits of MTK. In addition, by demonstrating the loss of the MTK-MCL joint activity but not MCL activity in both *mtkA* and *mtkB* mutants, we have shown that MTK is present in *M. extorquens* AM1. The fact that the *mtk* mutants grow normally on substrates other than C_1 compounds indicates that MTK in *M. extorquens* AM1 is specifically involved in the serine cycle.

The next gene in the cluster described here is *ppcA* (Fig. 1), encoding the acetyl-CoA-independent PEPC. This gene is separated from *mtkB* by over 400 bases. This region has been previously shown to be required for PEPC activity and to encode a polypeptide with a size of approximately 87 kDa, a size similar to the sizes of known PEPCs (2). The next gene in the cluster, *mclA*, is separated from *ppcA* by some 900 bases (8), and it is not known whether a coding region is present in this space.

Our current results suggest that the eight genes identified in the region described above are transcribed in groups, as we have identified promoter regions for *sgaA*, *hprA*, and *mclA* (8). The complementation data presented here suggest that a promoter is also present upstream of *mtkA*. Future work will focus on the transcriptional organization of this gene cluster.

Nucleotide sequence accession number. The sequence of 3,630 bp has been deposited with GenBank under accession no. L33465.

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